rco-3, a Gene Involved in Glucose Transport and Conidiation in *Neurospora crassa*

Lea Madi,[1] Sheila A. McBride, Lori A. Bailey and Daniel J. Ebbole

Department of Plant Pathology and Microbiology, Texas A&M University, College Station, Texas 77843

Manuscript received November 21, 1996
Accepted for publication February 14, 1997

ABSTRACT

Macroconidiation in *Neurospora crassa* is influenced by a number of environmental cues, including the nutritional status of the growing organism. Conidia formation is normally observed when the fungus is exposed to air. However, carbon limitation can induce conidiation in mycelia submerged in an aerated liquid medium. A mutant was previously isolated that could conidiate in submerged culture without imposing nutrient limitation and the gene responsible for this phenotype (*rco-3*) has now been cloned. RCO3 exhibits sequence similarity to members of the sugar transporter gene superfamily, with greatest similarity to glucose transporters of yeast. Consistent with this structural similarity, we find that glucose transport activity is altered in the mutant. However, growth of the mutant in media containing alternate carbon sources does not suppress conidiation in submerged culture. The properties of the mutant suggest that RCO3 is required for expression of glucose transport activity, glucose regulation of gene expression, and general carbon repression of development.

NEUROSPORA crassa is a filamentous fungus widely known for the ease with which it can be grown and genetically manipulated. It colonizes its substrate by radial growth of a vegetative mycelium composed of branched and interconnected multinucleate hyphae. With a linear growth rate on minimal medium of ~5 mm/hr, this initial phase of growth is rapidly completed. Further colonization is achieved by additional branching and growth of the substrate mycelium and by production of large numbers of orange, multinucleate spores called macroconidia (referred to as conidia throughout) (SPRINGER and YANOFSKY 1989; SPRINGER 1993). Conidiophore development is subject to complex environmental regulation. Rich nutrient sources, such as 2% peptone, can repress the extent of conidia formation. Regardless of medium composition, conidiation is a strictly aerial process on a solid substrate such as agar. However, in vigorously shaken liquid cultures in carbon-limited media, conidiation can occur on submerged mycelia (CORTAT and TURIAN 1974; TON THAT and TURIAN 1978; PLESOFSKY-VIG *et al.* 1983; GUIGNARD *et al.* 1984).

A number of genes have been isolated that are expressed during conidiation (BERLIN and YANOFSKY 1985; ROBERTS *et al.* 1988; ROBERTS and YANOFSKY 1989; HAGER and YANOFSKY 1990; BELL-PEDERSEN *et al.* 1992; LAUTER *et al.* 1992). One approach to identifying regulatory genes involved in conidiation is to examine mutants with altered expression of conidiation-specific genes (MADI *et al.* 1994). One such mutant displayed conidiophore development in liquid and in solid media (MADI *et al.* 1994). The altered gene was designated *rco-3* (regulator of conidiation genes-3). The *rco-3* mutant is partially resistant to L-sorbose, which restricts the radial growth rate of wild-type colonies. The messenger RNA (mRNA) levels of conidiation-specific genes in this strain were elevated during development in submerged culture (MADI *et al.* 1994).

Here we report the cloning and characterization of *rco-3*. The predicted sequence of the RCO3 protein reveals greatest similarity to glucose transporters of *Saccharomyces cerevisiae*. One explanation for the conidiation phenotype of the *rco-3* mutant is that reduced sugar uptake might result in carbon limitation and induce conidiation. However, examination of sugar transport activity, glucose-repressible gene expression, and the growth properties of mutant strains demonstrated a pleiotropic phenotype suggesting a broader role for RCO3 than expected for a sugar transporter. We suggest that RCO3 may function as a sensor of nutrient status.

MATERIALS AND METHODS

Strains and plasmids: Unless otherwise indicated, strains were obtained from the Fungal Genetics Stock Center (FGSC), Department of Microbiology, University of Kansas Medical Center, Kansas City, Kansas 66160-7420. Strain CH22-3 is a second generation backcross progeny of *rco-3* mutant CH10-7 (MADI *et al.* 1994) with wild-type ORS-6a (FGSC no. 2490) that was used for complementation experiments. We have given this allele the designation *rco-3*[1]. A cross of this strain with *fl* (fluffy) strain (FGSC no. 818) produced wild-

Corresponding author: Daniel J. Ebbole, Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX 77843. E-mail: dje0282@zeus.tamu.edu

[1]*Present address:* Department of Plant Pathology and Microbiology, Faculty of Agriculture, The Hebrew University of Jerusalem, Rehovot 76100, Israel.

TABLE 1

Summary of *rco-3* mutant alleles

Allele	Description of mutation
rco-3[1]	Seven base pair duplication (CATCAAA) results in frameshift following codon K222.
rco-3[2]	Insertion of hygromycin phosphotransferase gene between codons G342 and R343.
rco-3[3]	RIP-generated allele has 208 transition mutations that eliminate the uORF and cause the following changes to *rco-3* codons:

Q9Z[a]	A74V	M152I	L250F	G304R	P408L
P20L	M79I	A153V	A255V	G312S	L409F
G36S	A85T	G159R	H257Y	S330L	A413T
D38N	L90F	M171I	M261I	S340L	S415F
T39I	D96N	A175T	A262V	W341Z	M416I
G44S	H97Y	A177T	P276L	M348I	G429S
A47T	G114S	T183I	H277Y	M354I	M437I
M48I	Q118Z	T188I	R281C	C357Y	S441N
H54Y	A121I	A189V	T282I	Q358Z	A442I
S56L	G135S	A190I	T284I	Q376Z	A443T
T57I	V145I	P195L	M290I	A393T	D450N
Q65Z	Q149Z	L220F	L294F	S394L	L451F
P70L	S150F	H243Y	M301I	W395Z	V455I
					W459Z

[a] Q9Z indicates that glutamine (Q) at codon 9 is mutated to a stop codon (Z).

type strain 9483 and *rco-3*[1] strain 9481 obtained from the same ascus. The wild-type strains 74-OR23-1A (FGSC no. 987) and ORS-6*a* were used in crosses and for transformation in the *rco-3* gene inactivation analyses.

A 3.4-kilobase pair (kb) *Sma*I fragment containing *rco-3* was subcloned from cosmid X10:E5 of the Orbach/Sachs library (ORBACH and SACHS 1991; ORBACH 1994) into the *Sma*I site of pCB1004 (CARROLL *et al.* 1994), a vector with chloramphenicol resistance for bacterial selection and hygromycin resistance suitable for selection in fungi. A plasmid was designed to disrupt *rco-3* by homologous replacement. An interrupted *rco-3* gene was constructed by removing the 1.5-kb *Hpa*I fragment from this plasmid (pLM2) to create plasmid pLMDE3. The 1.5-kb *Hpa*I fragment contains the *Aspergillus nidulans trpC* promoter for expression of the *hph* gene conferring hygromycin resistance. This was inserted into pLMDE3 at the *Nae*I site at position 1805 to give pLMDE4. The plasmid was digested with *Sma*I and the 4.9-kb *Sma*I fragment containing the interrupted *rco-3* gene was used to transform protoplasts (VOLLMER and YANOFSKY 1986) of 74-OR23-1A to generate strain 9616. The *rco-3* gene interrupted with the hygromycin B resistance gene was given allele designation *rco-3*[5].

For repeat induced point (RIP) mutagenesis (SELKER and GARRETT 1988; SELKER 1990), strain 74-OR23-1*A* was transformed with pLM2 and an isolate containing a single copy was selected for crossing to ORS6*a*. A representative progeny strain with an *rco-3* phenotype was selected for sequence analysis and this allele is designated *rco-3*[3] (Table 1).

Culture conditions: Cultures were grown in 50 ml Vogel's minimal salts medium (DAVIS and DE SERRES 1970) supplemented with carbon sources and peptone as indicated, in 125-ml flasks. The cultures were grown for 16–20 hr on a rotary shaker at 200 rpm at 34°. Linear growth rates were measured using either synthetic cross medium or in Vogel's minimal salts medium in race tubes (DAVIS and DE SERRES 1970), as described (WHITE and WOODWARD 1995). Resistance to 2-deoxyglucose (Sigma) was measured by growth rate in race tubes during a 21-hr period the day following inoculation on Vogel's minimal salts medium containing 0.5% fructose supplemented with 0.05% 2-deoxyglucose. Conidiophore formation within the agar and sorbose resistance was examined from colonies grown in Vogel's medium with 0.05% glucose, 0.05% fructose, and 2% L-sorbose (DAVIS and DE SERRES 1970).

Nucleic acid extraction and analysis: Genomic DNA was isolated as described previously (VOLLMER and YANOFSKY 1986). Southern blot analyses were performed on nylon membranes (MagnaCharge, MSI) as described (SAMBROOK *et al.* 1989). The 3.4-kb *Sma*I fragment of pLM2 was used as a probe after random-primed labeling with α-[^{32}P]dCTP. RNA isolation and Northern blot analyses were performed as described previously (MADI *et al.* 1994). A cDNA clone for *N. crassa* actin was obtained from Dr. M. PLAMANN, Texas A&M University. *qa-2* and *con-10* genes have been described previously (ROBERTS *et al.* 1988; GEEVER *et al.* 1989).

DNA sequence analysis: The nucleotide sequence reported in this paper has been deposited in the Genbank sequence database under the accession no. U54768. The predicted amino acid sequence of RCO3 was used to search the databases using the BLAST search algorithm (ALTSCHUL *et al.* 1990) and alignments with individual proteins were performed using the BESTFIT program (DEVEREUX *et al.* 1984). A cDNA clone of *rco-3* was obtained from a lambda ZAP version II+ library constructed by Dr. R. H. GARRETT and sequenced on both strands. The PCR was used to amplify the *rco-3*[1] and *rco-3*[3] alleles for direct sequencing with primers. Several additional primers were synthesized for sequencing of the extensively mutated *rco-3*[3] allele.

Glucose uptake experiments: Glucose uptake experiments were performed following the procedure of SCHNEIDER and WILEY (1971). Mycelia were prepared by inoculating 5×10^8 conidia in 100 ml Vogel's minimal salts containing 2% glucose with 2% peptone. Conidia were germinated and grown with shaking for 16 hr at 30°. Cultures were harvested and washed by filtration. The washed cells were suspended in 500 ml Vogel's salts with peptone but without glucose. To measure glucose transport in glucose repressed cells the resuspended cells were washed three times with distilled water and stored at 4° until they were assayed. For preparing derepressed cells, the resuspended cells were incubated in medium without glucose for 2 hr at 30°. Cells were washed three times in distilled water before assaying. Uptake experiments were conducted at 30°. The experiments were initiated by adding *rco-3*[2] strain 9616 or the wild-type strain 74-OR23-1A cells at ~1 mg (dry weight)/ml to 125-ml flasks containing 25 ml Vogel's minimal salts with 0.25, 0.5, 1.0, 2.0, 5.0, or 40 mM glucose supplemented with 0.05 mCi/ml [^{14}C-U]glucose (251 mCi/mmol). The time course of uptake was measured by removing 3-ml samples of the cell suspension at 1-min intervals. Four time points were taken for each glucose concentration. The samples were expelled into 15 ml of ice-cold water, and the mixture was harvested by filtration. The cells were washed three times with 5 ml of cold distilled water, dried, individually weighed, and added to scintillation vials for radioactivity measurement. Glucose uptake per mg mycelium was plotted *vs.* time. The assay was linear over the 4 min period of the assay and coefficients of correlation (r^2) for linear plots were calculated for each assay (Cricket Graph 1.3.2, Cricket Software, Malvern, PA).

RESULTS

Cloning and sequence analysis of the *rco-3* gene: Mutants bearing the *rco-3*[1] allele are partially resistant to

the effects of sorbose on colony morphology (MADI *et al.* 1994). Wild-type *N. crassa* forms compact, opaque colonies with highly branched hyphae on sorbose-containing medium. The *rco-3*^{1} mutant forms spreading colonies with varying degrees of conidiophore formation within and above the agar. Cloning of the gene was accomplished by complementation of the *rco-3*^{1} mutant by protoplast transformation with pools of 96 cosmids. Complementation was observed as dense colonies formed in a background of spreading, somewhat transparent colonies. One pool of the initial 16 cosmid pools tested complemented the mutant phenotype repeatedly and a single cosmid was identified in a series of subpools. The complementing DNA was subcloned as a 3.4-kb *Sma*I fragment (see MATERIALS AND METHODS).

Previous genetic mapping showed that the *rco-3*^{1} mutation is linked to both *his-3* and the mating type locus, placing *rco-3* near the centromere of linkage group I (EBBOLE and MADI 1996). Restriction fragment length polymorphism (RFLP) analysis (METZENBERG *et al.* 1985) was used to map the *rco-3* clone near *mei-3* and the centromere and indicates that the complementing DNA maps to the *rco-3* locus, just to the left of the centromere (EBBOLE and MADI 1996).

A single long open reading frame interrupted by two introns with typical *N. crassa* intron consensus sequences (HAGER and YANOFSKY 1990) is predicted from sequence analysis of the complementing DNA (Figure 1). The spliced sequence would encode a 594 amino acid polypeptide containing 12 putative membrane-spanning domains (Figure 1) and other conserved sequences found in members of the sugar transporter superfamily (Figure 2). A cDNA clone was obtained and sequence analysis confirmed the location of the introns. The 5′ end of the cDNA clone was located 402 base pairs (bp) upstream of the predicted start codon for the *rco-3* structural gene. An upstream open reading frame capable of encoding a polypeptide of 22 residues begins 103 bp upstream of the *rco-3* coding region (Figure 1).

rco-3 has the greatest sequence similarity with hexose transporter genes of *S. cerevisiae.* Hxt1p (LEWIS and BISSON 1991) shares 35% identity and 60% similarity with the glucose transporter domain of the predicted RCO3 polypeptide (DEVEREUX *et al.* 1984). Alignment with SNF3 (CELENZA *et al.* 1988) of yeast revealed 37% identity and 58% similarity in the sugar transporter domain (residues 66 to 666 of SNF3). By comparison, the sugar transport domain of SNF3 displayed 29% identity and 53% similarity to Hxt1p. RCO3 is predicted to contain a C-terminal region consisting of a glutamine-rich stretch of amino acids followed by a serine- and threonine-rich region (Figure 1). A search (ALTSCHUL *et al.* 1990) of the *S. cerevisiae* genome revealed that no transporter homolog contained a C-terminal region similar to that of RCO3.

Analysis of *rco-3* mutants: We amplified and sequenced the *rco-3*^{1} allele from nucleotide position 318–2507 (Figure 1) and the only sequence difference from the wild-type copy was a 7-bp duplication of the sequence 5′-CATCAAA-3′ beginning at position 1438 (Figure 1; Table 1). This mutation shifts the reading frame and causes termination after an additional 89 codons.

To verify that insertion into the coding region of *rco-3* was sufficient to cause the phenotype observed in *rco-3*^{1} strains, a plasmid was designed to interrupt the wild-type copy of *rco-3* by homologous integration. A 1.5-kb *Hpa*I fragment containing the hygromycin phosphotransferase gene (*hph*) was cloned into the *Nae*I site of *rco-3* between the eighth and ninth membrane-spanning domains (Figure 1). This location (following glycine 342) interrupts one of the conserved motifs found in sugar transporters (Figure 2) and would be expected to eliminate sugar transport function. The *hph* gene is transcribed from the *A. nidulans trp* C promoter. There is no translational fusion between *rco-3* and *hph* and the insertion mutant is expected to produce a protein truncated after the eighth membrane-spanning domain. Homologous integration of DNA into the *N. crassa* genome occurs at a low frequency and is dependent on the length of DNA used to target integration (ASCH and KINSEY 1990). Two sorbose-resistant strains were identified by visual screening of ~300–400 primary transformants. These isolates produced conidiophores in shaken liquid cultures. Southern blot analyses confirmed that these two isolates contained homologous replacement of *rco-3* and both contained a single copy of the transforming DNA. Using an *rco-3* gene fragment as probe, *Sma*I digestion of chromosomal DNA yielded a fragment of 3.4 kb in wild type and 4.9 kb in the replacement strain, as expected. *Bam*HI digestion gave fragments of ~9.5 and 11 kb, respectively, and *Sac*I generated fragments of 2.7 and 4.1 kb, respectively (data not shown). Using the *hph* gene as probe, the same fragment sizes were detected in the gene replacement strain and no hybridization signal was detected in the wild type (data not shown). The two gene-replacement strains were sorbose resistant and conidiated in liquid shake culture, but neither strain conidiated within the agar of solid medium. This *rco-3* allele is designated *rco-3*^{2} (Table 1).

Although both *rco-3*^{1} and *rco-3*^{2} mutations were expected to result in null mutations, the *rco-3*^{2} strains were not identical in phenotype to *rco-3*^{1} strains. Unlike *rco-3*^{1}, *rco-3*^{2} strains did not form conidiophores within agar medium. One of the *rco-3*^{2} strains, designated strain 9616, was crossed to strain 9481 (*rco-3*^{1}). No progeny were recovered with a wild-type phenotype, however, there was a range of phenotypes observed among the progeny regardless of *rco-3* allele. The degree of sorbose resistance, conidiation within agar (for strains bearing the *rco-3*^{1} allele), and the amount of conidiation in liquid culture varied among the progeny. This suggests

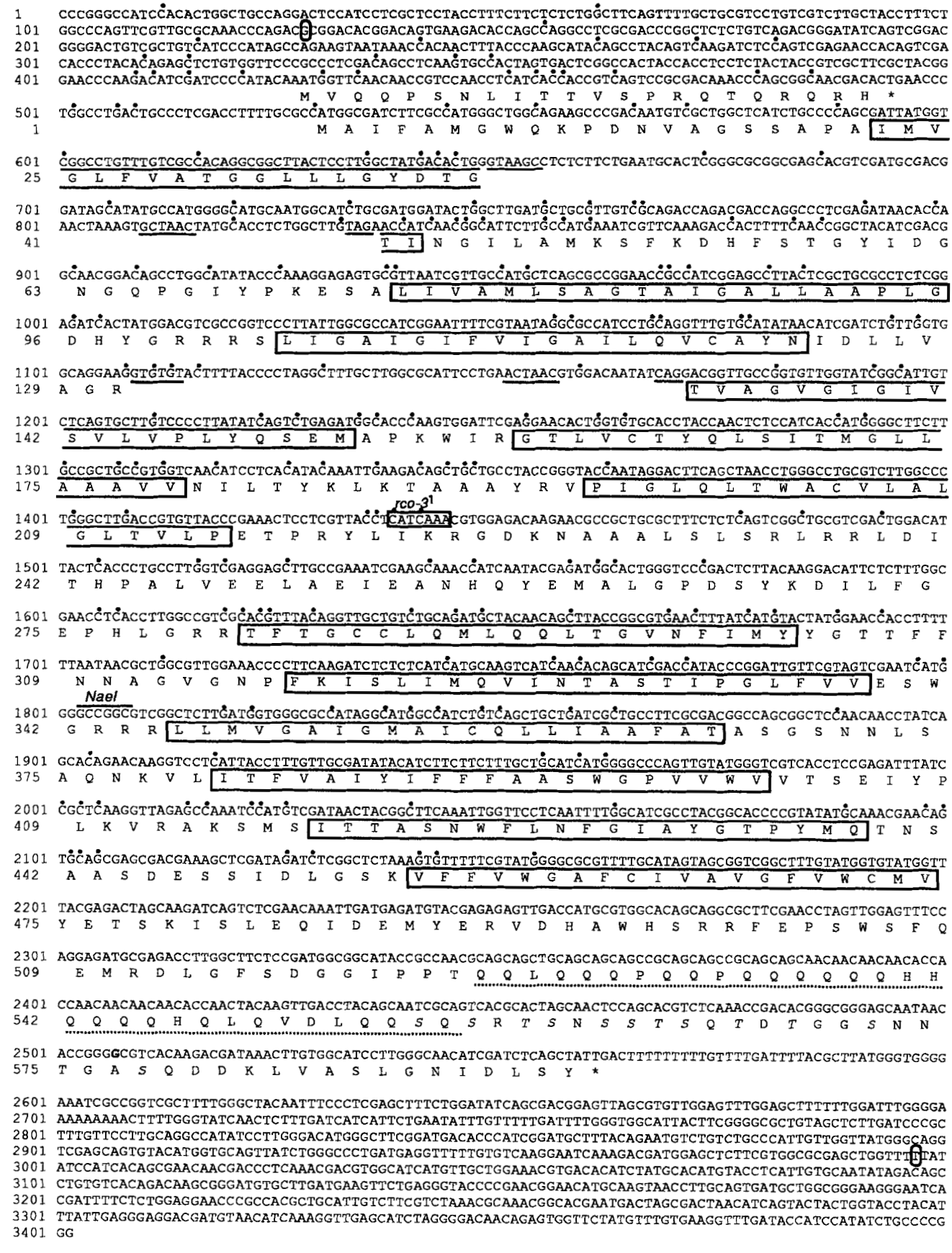

FIGURE 1.—Sequence analysis of the *rco-3* gene. The long open reading frame initiates at nucleotide 530 and is interrupted by introns of 188 and 62 nucleotides. Intron 5′, 3′, and acceptor site consensus sequences are underlined. The amino acid

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          96       214        338        474
RCO3      DHYGRR   PETPRYLI   VESWGRR    VYETSKISLEQI
          • ***    ** **      •   ***    • **    **
SNF3      DSYGRK   PESPRYYV   VEFFGRR    VYETKGLTLEEI
RGT2      DSYGRK   PESPRYYV   VDRIGRR    VYETRGLTLEEI
HXT1      DMYGRR   PESPRYLV   VERFGRR    VPETKGLTLEEV
HXT2      DMYGRR   PESPRFLV   VDKFGRR    VCETKGLTLEEV
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FIGURE 2.—Alignment of conserved regions of fungal transport proteins. SNF3 (CELENZA *et al.* 1988) and RGT2 are *S. cerevisiae* glucose transporter homologues that regulate HXT1 and HXT2 genes (ÖZCAN *et al.* 1996). HXT1 and HXT2 are hexose transporters (KRUCKEBERG and BISSON 1990; LEWIS and BISSON 1991). The locations of the conserved sequences in RCO3 are indicated by numbering above the RCO3 sequence. Residues conserved in all five transporter homologues are indicated by the asterisks.

that the phenotype of *rco-3* mutants can be partially suppressed by subtle differences in genetic background. However, none of the strains bearing the *rco-3*^{2} allele conidiated within the agar. Because of the difference between *rco-3*^{1} and *rco-3*^{2} alleles, it is possible that these alleles were not null mutations. The *rco-3*^{1} and *rco-3*^{2} alleles could produce polypeptides containing six and eight membrane-spanning domains, respectively, that might possess some activity.

Additional mutant alleles of *rco-3* were generated to examine the phenotype of *rco-3* null mutants. In *N. crassa*, duplicated genomic sequences are subject to mutation during mating by a process termed RIP mutation (SELKER and GARRETT 1988; SELKER 1990). RIP mutation results in numerous GC to AT transition mutations in both copies of the duplicated DNA, hence, RIP generated alleles are commonly null alleles. Roughly 50% of the nuclei bearing unlinked duplications undergo RIP during a cross (SELKER 1990). The wild-type strain 74OR-23-1A was transformed to hygromycin resistance with pLM2, a plasmid containing the *rco-3*$^+$ gene in a 3.4 kb *Sma*I fragment. The transformants were wild type in appearance. A transformant bearing a single ectopic copy of *rco-3* was crossed with wild-type ORS-6*a*. Fifty-four progeny that conidiated in liquid culture were recovered among the 200 viable progeny analyzed. A hygromycin-sensitive, sorbose-resistant progeny that conidiated in liquid medium was crossed to wild type. The progeny of this cross segregated 1:1 for conidiation in liquid medium. One of the mutant progeny (strain 9663) from this cross was chosen for Southern blot analysis and found to have a single copy of the *rco-3* gene (not shown). The *rco-3* gene from this strain was amplified by PCR and directly sequenced from nucleotide position 1–2220. RIP resulted in 208 transition mutations that caused 79 missense and nonsense mutations (Figure 1; Table 1). Based on the occurrence of termination codons at positions 9, 65, 118, 149, 341, 358, 376, 395, and 459, and other codon changes, we conclude that this RIP allele represents a null allele. The uORF in the RIP allele was also eliminated by mutation of the translation initiation codon and conversion of glutamine codons at positions 3, 18, and 20 to termination codons (Figure 1). The *rco-3*^{3} null allele is phenotypically indistinguishable from the *rco-3*^{2} allele (except for the hygromycin resistance associated with *rco-3*^{2}). We do not know why strains bearing the *rco-3*^{1} allele conidiate in agar while *rco-3*^{2} and *rco-3*^{3} strains do not.

Glucose uptake of the *rco-3*$^-$ strain: Since *rco-3* exhibited greatest sequence similarity to glucose transporters, we assessed glucose transport activity in the *rco-3* mutant. Previous glucose transport studies in *N. crassa* have demonstrated the existence of two transport systems (SCARBOROUGH 1970b; SCHNEIDER and WILEY 1971). A low-affinity (K_m = 20 mM) facilitated diffusion transport system is observed in glucose-repressed cells, and a high-affinity (K_m = 0.01 mM) active transport system is observed in glucose-limited cells (SCARBOROUGH 1970a,b; SCHNEIDER and WILEY 1971). The number of individual transport proteins that contribute to each transport system is not known. To distinguish high- and low-affinity transport we used a range of substrate concentrations from 0.25 to 40 mM glucose.

In glucose-grown wild-type cells, the low transport rate at 0.25–1 mM glucose relative to 40 mM glucose demonstrated that little high-affinity transport activity was present (Table 2) and that the majority of glucose transport activity observed in 40 mM glucose was due to the low-affinity system. In wild-type cultures that were starved for glucose for 2 hr prior to harvesting, transport was essentially saturated at 0.25–0.5 mM glucose, demonstrating the induction of the high-affinity transport system (Table 2). Thus, high- and low-affinity transport systems were readily distinguished.

Glucose transport activity of the *rco-3*^{2} strain was significantly altered from the wild type. Transport activity in glucose-repressed hyphae was greater than in the wild type at low substrate concentrations (Table 2), indicating that high-affinity transport was partially induced in the presence of 2% glucose. In contrast, glucose transport activity was much lower than the wild type at high substrate concentration, showing that low-affinity transport activity was largely absent.

Following glucose starvation, high-affinity transport

sequences of 12 putative membrane-spanning domains deduced by alignment with fungal transport proteins are boxed. A glutamine-rich region beyond the region of homology to fungal transport proteins is indicated by the dotted underline. A cluster of serine and threonine residues following the glutamine-rich region are indicated by italic type. The 5′ and 3′ ends of the cDNA clone are indicated by the circled nucleotides at positions 128 and 2997. An upstream open reading frame of 22 residues initiates at position 428. The *rco-3*^{1} mutant gene was sequenced from position 318 to 2507. The only sequence change was a seven base pair duplication indicated by boxed nucleotides at positions 1438–1444. The nucleotide sequence of the *Nae*I site used to construct the *rco-3*^{2} insertion mutation is overlined. The position of transition mutations caused by RIP mutagenesis from nucleotides 1 to 2220 are indicated as dots over the nucleotide sequence.

TABLE 2

Glucose transport in the wild-type (74-OR23-1VA) and *rco-3*^{2} (9616) strains

Glucose (mM)	Glucose-grown cells				Glucose-starved cells			
	Wild type		*rco-3*		Wild-type		*rco-3*	
	Uptake	r^{2a}	Uptake	r^2	Uptake	r^2	Uptake	r^2
0.25	1.0	0.96	5.0	0.99	48	0.99	23	0.99
0.5	2.5	0.97	7.8	0.99	56	0.99	24	0.98
1.0	4.0	0.95	7.1	0.99	61	0.99	20	0.99
2.0	8.0	0.95	7.1	0.99	68	0.99	26	0.99
5.0	13	0.99	6.8	0.98	54	0.99	21	0.99
40	61	0.91	11	0.96	63	0.99	18	0.94

Values are nmol glucose transported/min/mg.

[a] Correlation coefficient for linear uptake during the time course.

in the *rco-3*^{2} mutant was elevated. However, the level of high-affinity transport was two- to threefold lower than in wild type (Table 2). This suggests that the activities of one or more high-affinity glucose transporters are reduced in the mutant. Thus, *rco-3* mutants appear to be deficient in both high- and low-affinity transport activity.

Growth properties of the *rco-3* mutants: Based on glucose transport studies it seemed possible that conidiation of the *rco-3* mutants in liquid medium resulted from starvation for glucose imposed by the transport defect. We noted that, on solid medium, strains bearing any of the three *rco-1* alleles grew at the rate of wild type or were only slightly reduced in growth rate on glucose (5–30%) (data not shown). This was true even for the *rco-1*^{1} strains that can conidiate in agar medium. However, conidiation in liquid shake cultures caused all of the *rco-3* mutants to increase biomass very slowly compared to wild-type strains. Therefore, we measured linear growth rates of the wild-type and *rco-3*^{2} mutant on solid media. Growth of *rco-3*^{2} mutant on 2% glucose containing medium was reduced roughly 20% relative to wild type (Table 3). This suggests that the strong reduction in glucose transport activity in 2% glucose-grown cells (Table 2) may be severe enough to limit growth rate of the *rco-3* mutants on agar medium. There was no significant difference in relative growth rate of *rco-3* strains when grown on media containing 2% fructose, glycerol, xylose, or 0.05% glucose.

TABLE 3

Growth properties of *rco-3*^{2} mutant and wild-type strains

Carbon source	Linear growth rate[a]		Phenotype[b]	
	rco-3	wt	*rco-3*	wt
2% Glucose	4.1 ± 0.4	4.8 ± 0.1	C	M
2% Fructose	4.4 ± 0.0	4.5 ± 0.3	C	M
2% Xylose	4.2 ± 0.2	4.0 ± 0.4	C	M
2% Glycerol	2.9 ± 0.1	2.8 ± 0.3	C	M
2% Peptone[c]	4.4 ± 0.2	5.4 ± 0.3	M	M
0.05% Glucose	3.1 ± 0.0	3.1 ± 0.1	C	M/C

[a] Linear growth rate on agar media determined in race tubes; values are means ± SD in mm/hr.

[b] Predominantly conidiation during growth of culture (C), mycelial growth without conidiation (M), or initially mycelial then conidiating (M/C) in liquid shake cultures.

[c] 2% Peptone + 2% glucose medium.

If the primary role of *rco-3* is limited to glucose transport the *rco-3* mutants would not be expected to have a discernable phenotype when glucose is absent from the medium. The wild type grows as filamentous hyphae in minimal medium with 2% glucose (Figure 3). The *rco-3* mutant strains display a budding pattern of growth observed in conidiophores (Figure 3). In media containing 2% fructose (Figure 3), xylose, or glycerol, wild-type strains also grow in a filamentous form (Table 3). However, *rco-3* mutants produce budded cells resembling conidiophores regardless of carbon source (Figure 3; Table 3). In media containing 2% glucose and 2% peptone, hyphae of both the wild-type and *rco-3* strains exhibited a slightly irregular hyphal morphology (Figure 3). However, conidiation of the *rco-3* strains was suppressed in peptone medium. Wild-type cultures grown in 0.05% glucose were initially mycelial but then formed limited numbers of conidiophores within 24 hr (Table 3). The *rco-3* mutants invariably formed conidia earlier and in greater abundance than the wild type.

Glucose regulation of gene expression in *rco-3* mutants: Other sorbose-resistant mutants are known in *N. crassa*, including *dgr1-4* that are resistant to both sorbose and 2-deoxyglucose (ALLEN *et al.* 1989). We found that the *rco-3* mutant strains were also resistant to 2-deoxyglucose. On 0.5% fructose minimal medium the linear growth rate was 4.1 ± 0.5 mm/hr for wild type (74-OR23-1A) and 4.3 ± 0.2 mm/hr for *rco-3*^{2} strain 9616. Supplementation of the medium with 0.05% 2-deoxyglucose completely blocked growth of the wild type during the first 48 hours following inoculation. However, no growth lag was observed for strain 9616 and it grew at 2.6 ± 0.2 mm/hr. Thus, the *rco-3* mutant was able to use fructose as a carbon source for growth in the presence of 2-deoxyglucose.

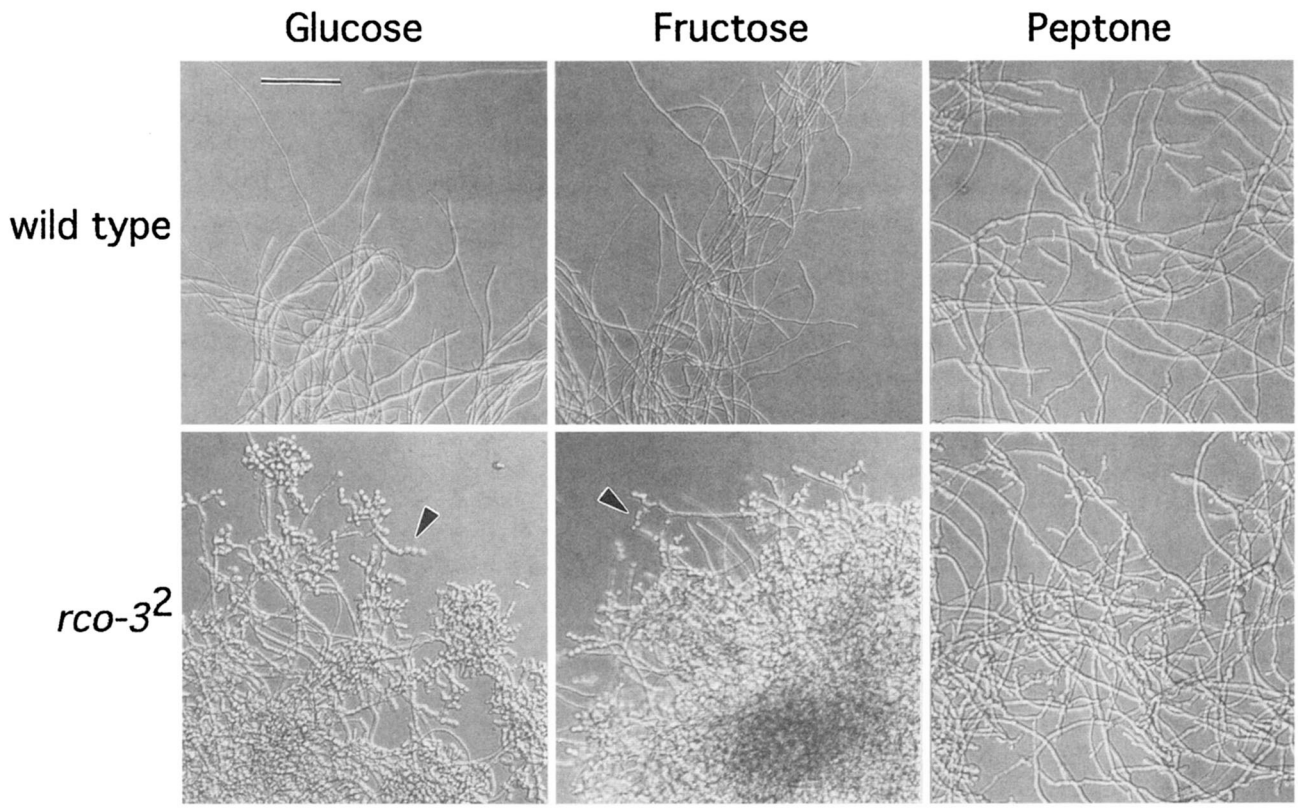

FIGURE 3.—Phenotype of wild-type and *rco-3* strains grown in liquid media. Wild-type 74-OR23-1A and *rco-3*^{2} strain 9616 were grown in minimal medium with 2% glucose or 2% fructose and in 2% peptone plus 2% glucose medium. Wild type grows as hyphal filaments in minimal medium with glucose or fructose. Strain 9616 has the same phenotype as *rco-3*^{1} and *rco-3*^{3} strains and forms conidial chains in these media (arrowheads). Both wild-type and *rco-3* strains have slightly swollen and irregular hyphal morphology in 2% peptone medium, but conidiation is absent in both the wild-type and the *rco-3* strains. All cultures were observed at ×100 power with a Zeiss Axioskop microscope. Bar, 115 μm.

Some of the previously characterized 2-deoxyglucose-resistant mutants were found to be defective in glucose repression (ALLEN *et al.* 1989). Therefore, we next examined the expression of a glucose-repressible gene involved in quinic acid utilization *qa-2* and the developmentally regulated gene *con-10*. The *rco-3*^{1} and wild-type strains were grown in minimal medium with 2% glucose and were transferred to medium lacking glucose for 2 hr. These media contained 0.1% quinic acid to activate the *qa-2* gene (GEEVER *et al.* 1989). Glucose repression of *qa-2* in wild type was readily apparent. However, *qa-2* was not repressed by glucose in the *rco-3* strain (Figure 4). In the wild type, *con-10* expression was not detected in 2% glucose or in response to glucose starvation (Figure 4). *con-10* mRNA was abundant in the *rco-3*^{1} strain and glucose starvation did not further elevate expression. We were unable to detect *rco-3* mRNA in these same Northern blots (not shown).

DISCUSSION

rco-3 was selected following UV mutagenesis as a mutant that had elevated basal expression of a conidiation-specific gene *con-10* (MADI *et al.* 1994). The selection strategy required growth of mutagenized cells in submerged agar culture that blocks conidiation in the wild type. Selection of a mutant that can conidiate when embedded in agar is therefore not surprising. It is interesting that strains bearing the *rco-3*^{1} allele can conidiate in agar, but *rco-3*^{2} and *rco-3*^{3} do not. Further characterization of this difference between alleles is warranted.

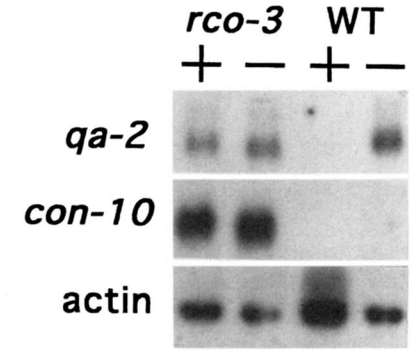

FIGURE 4.—Gene expression and glucose repression in strains 9481 (*rco-3*^{1}) and 9483 (wild type). Panels from Northern blot analyses with 20 μg of total RNA from glucose repressed cells (+) and cells starved for glucose for 2 hr (−) in minimal medium. Blots were probed with the glucose-repressible gene *qa-2*, the developmentally regulated gene *con-10*. The actin gene was used to indicate the relative amount of RNA in each lane.

We are investigating how environmental signals are sensed and coordinated to signal induction of conidiation. One key environmental signal is the availability of readily assimilated carbon sources. *rco-3* mutants are able to bypass the normal requirement for nutrient deprivation to initiate development in liquid cultures. Sequence analysis of *rco-3* revealed that the gene is a member of the superfamily of sugar transporters. Although we do not know the substrate specificity of RCO3 it appears to be most similar to hexose transporters of *S. cerevisiae.* The high degree of similarity in the 12 membrane-spanning domains characteristic of sugar transporters, as well as other sequences conserved in fungal transport proteins, suggest that RCO3 could function as a membrane-bound transporter.

The number of individual glucose transporters that contribute to high- and low-affinity transport in *N. crassa* is not known; however, hexose transporters comprise a large gene family in *S. cerevisiae* (ÖZCAN *et al.* 1996). A substantial reduction of low-affinity glucose transport was observed in the *rco-3*^{2} mutant, suggesting the possibility that RCO3 represents the major contributor to low-affinity transport activity. High-affinity transport was also reduced twofold. Thus, if RCO3 acts as a transporter, it may have unique properties and function in both high- and low-affinity transport. However, in *S. cerevisiae,* high- and low-affinity transport activity is thought to be the result of different high- and low-affinity transporters rather than individual transporters that can function with both high- and low-affinity (COONS *et al.* 1995). Alternatively, RCO3 might interact with other glucose transport proteins to affect their activities. A third possibility is that RCO3 is not primarily a transporter, but functions in glucose sensing.

Mutation of *rco-3* alters glucose regulation: The initial transport of 2-deoxyglucose in the *rco-3* mutants should be fairly efficient since the high-affinity glucose transport system would be active in the absence of high levels of external glucose. Phosphorylated 2-deoxyglucose accumulates in *S. cerevisiae* and *N. crassa* (ALLEN *et al.* 1989) and has been shown to act as a gratuitous activator of glucose repression in *S. cerevisiae* (ZIMMERMANN and SCHEEL 1977). Fructose is transported by a distinct transport system and is not appreciably transported by the glucose transport systems in *N. crassa.* The fructose transport system is repressed by glucose and growth on fructose is inhibited by the presence of 2-deoxyglucose (ALLEN *et al.* 1989). The partial growth inhibition of the *rco-3*^{2} strain on fructose plus 2-deoxyglucose medium suggests that 2-deoxyglucose is transported into the cell and that fructose transport is not repressed by 2-deoxyglucose. This suggests that glucose sensing and subsequent repression of nonglucose transporters rather than transport of 2-deoxyglucose is defective. In addition, the *qa-2* gene is not repressed by glucose in the *rco-3* mutants. These findings suggest that glucose repression may be generally defective in *rco-3* mutants. However, low-affinity glucose transport in the *rco-3* mutant is virtually absent and high-affinity transport is partially activated in the mutant grown on 2% glucose. It is possible that the loss of glucose repression of *qa-2* is primarily due to the reduced low-affinity glucose transport activity. Additional studies are needed to prove whether the loss of glucose repression is due to sensing of glucose levels or a reduction in glucose or 2-deoxyglucose transport.

***rco-3* is required for carbon repression of conidiation:** Wild-type strains do not initiate conidiophore development in a liquid medium containing 2% glucose, fructose, xylose, or glycerol as carbon source. Therefore, conidiation is repressed in wild type, not only by glucose, but also by other carbon sources regardless of external glucose concentration. The failure of any of these carbon sources to suppress the submerged conidiation phenotype of the *rco-3* mutants is a strong indication that RCO3 does not act solely as a glucose transporter. One possibility is that RCO3 functions as part of a transport complex and might influence transport of many compounds. However, the fact that *rco-3* mutants grow as fast as wild type on fructose, xylose, glycerol, and 0.05% glucose suggests that transport of these compounds is not limiting for growth. If RCO3 functions as a sensor of glucose, *rco-3* mutants may be incapable of proper signaling of carbon status regardless of carbon source. RCO3 may provide input into separate pathways controlling glucose metabolism and conidiation, each subject to additional controls. For example, peptone represses conidiation in the *rco-3* mutants but does not suppress the defect in glucose transporter activity. Genetic analysis of the relationships between *rco-3* and other known mutations conferring 2-deoxyglucose and sorbose resistance in *N. crassa* are needed to help identify the pathways involved in glucose-specific regulation of gene expression and signaling of conidiation. Like *rco-3, dgr-2 and dgr-3* both map near the centromere of linkage group I (ALLEN *et al.* 1989). Preliminary examination shows that these two mutants do not conidiate in liquid culture (D. J. EBBOLE, unpublished observation).

RCO3 as glucose sensor: Both high- and low-affinity glucose transport activities appear to be altered in the *rco-3* mutant. RCO3 may regulate expression of genes for both high- and low-affinity glucose transport. Recently, two sugar transporter homologues in *S. cerevisiae,* SNF3 and RGT2, were proposed to act as glucose sensors that control hexose transporter gene expression (ÖZCAN *et al.* 1996). These genes are expressed at low levels (ÖZCAN and JOHNSTON 1995; ÖZCAN *et al.* 1996) as apparently is true for *rco-3* based on the lack of detected mRNA in Northern blot experiments. Sequence analysis of a cDNA clone demonstrated that *rco-3* mRNA contains an uORF that likely reduces translation efficiency. Interestingly, short uORFs initiate 34 bp upstream of the predicted SNF3 start codon and 26 bp

upstream of the predicted RGT2 start codon. It is possible that expression of these genes is also modulated at the translational level.

In addition to the glucose transporter homology, the RCO3 polypeptide is predicted to contain a unique C-terminal region. SNF3 and RGT2 (884 and 763 amino acids, respectively) contain C-terminal domains that are significantly larger than the C-terminal tail of RCO3. Both of the yeast protein C-terminal domains contain a short conserved sequence motif that occurs twice in SNF3 and once in RGT2 (ÖZCAN *et al.* 1996). There is no sequence similarity between the C-terminal tails of RCO3 and the yeast glucose sensors. The roles of these domains remain to be elucidated; however, SNF3 function appears to depend on the C-terminal domain (MARSHALL-CARLSON *et al.* 1990).

SNF3 is proposed to act as a sensor of low-glucose levels and RGT2 is proposed to function as a high-glucose sensor. *rco-3* mutants appear capable of sensing low-glucose levels since high-affinity glucose transport could be activated by glucose limitation. The inability to activate low-affinity transporters suggest that, if RCO3 is a glucose sensor, it may be a sensor of high-glucose levels. This would be consistent with its role in repressing conidiation. Further analysis of the role of *rco-3* in glucose transport, glucose repression, and repression of conidiophore development will help clarify the function of *rco-3* and provide insight into the relationship between carbon metabolism and the physiological control of conidiation in *N. crassa.*

The authors thank Dr. MIKE PLAMANN for providing the *N. crassa* actin cDNA clone and Drs. TOM ADAMS, CHARLES YANOFSKY, and PETER MARGOLIS for comments on the manuscript. We thank Drs. YIGAL HENIS and ODED YARDEN for helpful suggestions. These studies were supported by a Public Health Service grant (R29 GM-47907) to D.J.E.

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Communicating editor: J. J. LOROS